

1. Protocols used for the flower-specific cDNA array

RNA amplification

This protocol generates *in vitro* transcribed RNA for microarray hybridizations. In a first step polyA-RNA is amplified by *in vitro* transcription (according to Eberwine and collaborators).

METHOD:

I: cDNA synthesis

1: Mix:	Total RNA	3-10 µg
	T7dT primer (0.5µg/µl)	1 µl
	Control RNA (optional)	x µl
	RNase-free H ₂ O	to 12 µl

2: Incubate at 70°C for 10 min. Quick-chill on ice. Collect the contents of the tube by quick centrifugation.

3: Add:	5X First Strand Buffer	4 µl
	0.1 M DTT	2 µl
	dNTP-mix (10 mM each)	1 µl

4: Incubate at 37°C for 2 min to equilibrate the temperature.

5: Add 1 µl of Superscript II and mix gently.
Final volume of the 1st strand reaction: 20 µl

6: Incubate at 37°C for 1 hour. Put on ice.

7: Second strand synthesis.

Add:	H ₂ O	92 µl
	5X Second Strand Buffer	30 µl
	dNTP-mix (10 mM each)	3 µl
	<i>E.coli</i> DNA Polymerase I	4 µl
	<i>E.coli</i> RNase H	1 µl

Final volume of the 2nd strand reaction: 150 µl

8: Incubate at 16°C for 2 hours. Do not let the temperature rise above 16°C.

9: Add 10 µl of 0.5 M EDTA to stop the reaction.

II: Purification of cDNA

1: Transfer reaction mix into a phase-lock gel tube (spin tubes briefly before use to collect the gel on the bottom).

- 2:** Add 160 μ l of phenol:chloroform:isoamyl alcohol (25:24:1). Mix thoroughly, but do not vortex. Spin 5 min at 14k rpm (12,000-16,000 \times g).
- 3:** Pipet off aqueous layer and place in a fresh tube.
- 4:** Add an equal volume (\sim 160 μ l) of 5M NH_4OAc etate.
- 5:** Add 2.5x volumes of 100% EtOH (\sim 800 μ l). Add 1 μ l of Linear Polyacrylamide. Mix and spin for 5 min at 14k rpm, RT.
- 6:** Remove supernatant carefully and wash pellet with 500 μ l of 80% EtOH. Do not vortex.
- 7:** Spin for 5 min at 14k rpm.
- 8:** Repeat the wash step.
- 9:** Dry pellet in a speed-vac.
- 10:** Resuspend pellet in 10 μ l of RNase-free H_2O .

III: *In vitro* transcription

Use Ambion's Megascript T7 kit. Set up reaction at room temperature.

- 1:** Mix the following:

RNase-free H_2O	3 μ l
ATP solution (75 mM)	2 μ l
GTP solution (75 mM)	2 μ l
CTP solution (75 mM)	2 μ l
UTP solution (75 mM)	2 μ l
10X reaction buffer	2 μ l
cDNA from previous step	5 μ l
<u>T7 Enzyme</u>	<u>2 μl</u>
FINAL VOLUME:	20 μ l

- 2:** Incubate the reaction at 37°C for 6 hours to overnight.
- 3:** Clean up the RNA on a Qiagen RNeasy column:
 - Add 80 μ l of RNase-free water
 - Add 350 μ l of buffer RLT to the sample (add β -ME to buffer RLT before use; 10 μ l β -ME / 1 ml buffer).
 - Add 250 μ l 100% EtOH to the sample. Mix well by pipetting.
 - Transfer sample (700 μ l) to an RNeasy mini spin column.
 - Centrifuge for 15 sec at full speed.
 - Transfer RNeasy column to a new 2-ml collection tube (supplied in kit)
 - Pipet 500 μ l RPE onto the column. Centrifuge for 30 sec at maximum speed.
 - Pipet 500 μ l RPE onto the column. Centrifuge for 2 min at maximum speed. Discard flow-through.

- Centrifuge at full speed for 1 min.
 - Transfer RNeasy column into a 1.5 ml collection tube.
 - Add 30 µl of RNase-free water directly onto the RNeasy membrane.
 - Centrifuge for 1 min at full speed.
 - Repeat elution step with another 30 µl of water, into the same collection tube.
- Final volume: 50-60 µl.

4: Run 2 µl of the RNA on a 1% agarose gel or run an aliquot on a Bioanalyzer. Determine RNA concentration and calculate total yield. The transcription reaction should result in 30-50 µg of RNA. Store RNA at -80°C until use.

MATERIALS AND REAGENTS:

Invitrogen:

E.coli Ribonuclease H (Cat. No. 18021071); 120 units, enough for 60 rxns
E.coli DNA Polymerase I (Cat. No.18010025); 1000 units, enough for 25 rxns
 Superscript II (Cat. No.18064071); 4x10,000 units, enough for 200 rxns
 dNTP set (100 mM) (Cat. No. 10297018)

Ambion:

Megascript T7 kit (Cat. No. 1334), enough for 40 rxns

Eppendorf:

Phase Lock Tubes, Gel Light (200), 1.5 ml tubes (Cat. No. 0032 007.961)

Qiagen:

RNeasy Mini Kit (250) (Cat. No. 74106)

Sigma:

Linear Polyacrylamide (Cat. No. 5-6575)

Various sources:

T7dT primer (PAGE purified):

5' - TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG TTT
 TTT TTT TTT TTT TTT TTN N-3'

Phenol:chloroform:isoamyl alcohol (25:24:1).

SOLUTIONS:

5x Second Strand Buffer (store at -20°C):

100 mM Tris-HCl pH 6.9
 450 mM KCl
 23 mM MgCl₂
 50 mM (NH₄)SO₄
 0.75 mM β-NAD⁺

Indirect Aminoallyl-based Probe Labeling Protocol

In-vitro transcribed (IVT) RNA prepared using the RNA Amplification protocol is used for generating the probe. The protocol follows the instructions from the Atlas Glass Fluorescent Labeling Kit (Clontech), except that the cDNA synthesis control and the coupling reaction control oligo are not used.

Avoid exposure of Cy-dyes to light: use amber Eppendorf tubes, wrap them in aluminum foil, turn off bench lights, work under dim light if possible.

1.) Mix (in 0.5 ml tubes for the thermal cycler):

IVT RNA	5 µg
Random hexamer primer (0.2µg/µl)	2.5 µl
<u>H₂O</u>	<u>X µl</u>
FINAL VOLUME	25 µl

2.) Prepare a master mix that is sufficient for the number of reactions to be performed:

Mix per reaction:	10 µl	5X cDNA synthesis buffer
	5 µl	10X dNTP mix
	7.5 µl	H ₂ O
	2.5 µl	MMLV Reverse Transcriptase (200U/µl)

3.) Put RNA/primer tubes in the thermal cycler and follow the protocol of the Atlas kit precisely. Synthesis reaction is performed at 37°C. Program in thermal cycler is:

70°C, 5 min (as soon as 37°C is reached, add 25 µl of master mix to each tube)

37°C, 1 hour

70°C, 5 min (as soon as 37°C is reached, add 0.5 µl of RNaseH to each tube, mix well by pipetting, spin down for a few seconds, and return to thermal cycler)

37°C, 20 min

4.) Stop reaction and clean up the cDNA according to the Atlas kit protocol, steps A11 through A18.

5.) Perform fluorescent dye coupling and probe purification according to the Atlas kit protocol, steps B1 through C9.

6.) Mix the corresponding Cy3 and Cy5 purified probes (~100 µl each)

MATERIALS AND REAGENTS

Clontech: Atlas Glass Fluorescent Labeling Kit (Cat No. K1037-1)

Roche: Primer random p(dN)₆ (Cat. No. 1 034 731).

Eppendorf: Standard Tube, amber, 1.5 ml (Cat No. 22 36 413-8).

Microarray hybridization

The following is a protocol for the hybridization of labeled cDNA to cDNA arrays. This protocol works well for arrays printed on poly-lysine or on Corning GAP slides. The amount of hybridization solution is sufficient to cover a 25X25mm array.

I: Slide Hybridization

- 1:** Dry down purified probes in a speed-vac. Resuspend pellet in 1.5 μ l 10 mM EDTA. Heat up sample at 95°C for 1 min.
- 2:** Add 15 μ l of Ambion SlideHyb Buffer #1 (pre-warmed at 70°C).
- 3:** Incubate at 95°C for 2 min. Spin for 30 sec. Incubate at 95°C for a few seconds.
- 4:** Apply probe (16.5 μ l) to one edge of the array and carefully lay down cover slip without capturing air bubbles underneath. Avoid scratching the array. Put slide on the pre-warmed bottom part of a hybridization chamber (60-70°C).
- 5:** Distribute a total of 17 μ l of 3XSSC in small drops left and right from the cover slip.
- 6:** Close the hybridization chamber and incubate it in a water bath for 12-16 h at 50°C. Cover the water bath with a lid or with aluminum foil, to avoid any light exposure (high temperatures accelerate destruction of the fluorophores).
- 7:** Put a glass dish and 1 liter of water in a 60°C incubator.

VI.) Slide Washes

- 1:** Prepare 1 liter of the first wash buffer: 1XSSC, 0.2% SDS (make up with pre-warmed water). Put a slide rack in the bottom of the pre-warmed glass dish and add the wash buffer. When the temperature of the wash buffer is ~50°C dip a slide into the wash buffer, turn it up-side-down and shake it gently until the cover slip comes off. Insert slide into the slide tray without taking it out of the wash solution. Cover the dish with aluminum foil and gently shake it for 10 min.
- 2:** Transfer the slide rack to a dish with 1 liter of 0.1XSSC, 0.2% SDS (room temp). Cover the slide chamber with aluminum foil and gently shake it for 10 min.
- 3:** Transfer the slide rack to a dish with 1 liter of 0.1XSSC (room temp). Shake dish gently for 1 min.

4: Take the slides out of the last wash buffer and centrifuge them for 3 min at 50 x g (~500 rpm). Store the slides so that they are protected from light. We usually scan the slides right away since we have found that the fluorescent signals (Cy5, in particular) start to fade rapidly.

MATERIALS AND REAGENTS

Ambion: SlideHyb Buffer #1 (Cat No. 8861)

20xSSC: 3 M NaCl (175.3g)
0.3 M Na-citrate (88.2g)
add H₂O to 1 l and adjust pH to 7 with NaOH

10% (w/v) SDS

2. Protocols used for the oligonucleotide array

RNA amplification and labeling of RNA probes

This protocol generates dye-labeled antisense RNA probes for microarray hybridizations. In a first step polyA-RNA is amplified by *in vitro* transcription (according to Eberwine and collaborators). During *in vitro* transcription aminoallyl-UTP is incorporated into the newly synthesized RNA. NHS-ester dyes are then directly coupled to the modified bases in a simple chemical reaction.

METHOD:

Protect all dye-containing solutions from light to prevent photo bleaching. We are using total RNA purified on Qiagen RNeasy columns as starting material but other RNA isolation methods may work as well.

I: cDNA synthesis

1: Mix:	Total RNA	3-10 µg
	T7dT primer (0.5µg/µl)	1 µl
	Control RNA (optional)	x µl
	RNase-free H ₂ O	to 12 µl

2: Incubate at 70°C for 10 min. Quick-chill on ice. Collect the contents of the tube by quick centrifugation.

3: Add:	5X First Strand Buffer	4 µl
	0.1 M DTT	2 µl
	dNTP-mix (10 mM each)	1 µl

4: Incubate at 37°C for 2 min to equilibrate the temperature.

5: Add 1 µl of Superscript II and mix gently.
Final volume of the 1st strand reaction: 20 µl

6: Incubate at 37°C for 1 hour. Put on ice.

7: Second strand synthesis.

Add:	H ₂ O	92 µl
	5X Second Strand Buffer	30 µl
	dNTP-mix (10 mM each)	3 µl
	<i>E.coli</i> DNA Polymerase I	4 µl
	<i>E.coli</i> RNase H	1 µl

Final volume of the 2nd strand reaction: 150 µl

8: Incubate at 16°C for 2 hours. Do not let the temperature rise above 16°C.

9: Add 10 µl of 0.5 M EDTA to stop the reaction.

II: Purification of cDNA

1: Transfer reaction mix into a phase-lock gel tube (spin tubes briefly before use to collect the gel on the bottom).

2: Add 160 µl of phenol:chloroform:isoamyl alcohol (25:24:1). Mix thoroughly, but do not vortex. Spin 5 min at 14k rpm (12,000-16,000 x g).

3: Pipet off aqueous layer and place in a fresh tube.

4: Add an equal volume (~ 160 µl) of 5M NH₄OAcetate.

5: Add 2.5x volumes of 100% EtOH (~ 800 µl). Add 1 µl of Linear Polyacrylamide. Mix and spin for 5 min at 14k rpm, RT.

6: Remove supernatant carefully and wash pellet with 500 µl of 80% EtOH. Do not vortex.

7: Spin for 5 min at 14k rpm.

8: Repeat the wash step.

9: Dry pellet in a speed-vac.

10: Resuspend pellet in 10 µl of RNase-free H₂O.

III: *In vitro* transcription

Use Ambion's Megascript T7 kit and aminoallyl-UTP. Set up reaction at room temperature.

1: Mix the following:

RNase-free H ₂ O	2.5 µl
ATP solution (75 mM)	2 µl
GTP solution (75 mM)	2 µl
CTP solution (75 mM)	2 µl
UTP solution (75 mM)	1 µl
aa-UTP solution (50 mM)	1.5 µl
10X reaction buffer	2 µl
cDNA from previous step	5 µl
<u>T7 Enzyme</u>	<u>2 µl</u>
FINAL VOLUME:	20µl

2: Incubate the reaction at 37°C for 6 hours to overnight.

3: Clean up the RNA on a Qiagen RNeasy column:

- Add 80 µl of RNase-free water

- Add 350 µl of buffer RLT to the sample (add β-ME to buffer RLT before use; 10 µl β-ME / 1 ml buffer).
- Add 250 µl 100% EtOH to the sample. Mix well by pipetting.
- Transfer sample (700 µl) to an RNeasy mini spin column.
- Centrifuge for 15 sec at full speed.
- Reload column with flow-through and spin for 15 sec at full speed. This step may increase the RNA yield.
- Transfer RNeasy column to a new 2-ml collection tube (supplied in kit)
- Add 500 µl of phosphate wash buffer. (Don't use Qiagen's buffer RPE since it contains Tris that might interfere with the labeling reaction). Centrifuge for 15 sec at 14 k (>8,000 x g), and discard flow-through
- Pipet 500 µl of phosphate wash buffer onto the column. Centrifuge for 2 min at maximum speed. Discard flow-through.
- Centrifuge at full speed for 1 min.
- Transfer RNeasy column into a 1.5 ml collection tube.
- Add 30 µl of RNase-free water directly onto the RNeasy membrane.
- Centrifuge for 1 min at full speed.
- Repeat elution step with another 30 µl of water, into the same collection tube. Final volume: 50-60 µl.

4: Run 2 µl of the RNA on a 1% agarose gel or run an aliquot on a Bioanalyzer. Determine RNA concentration and calculate total yield. The transcription reaction should result in at least 30-50 µg of RNA. Store RNA at -80°C until use.

IV. RNA labeling

Important: Make sure, to never over-dry the RNA in the following steps. Precipitated RNA will appear as colored speckles on the membrane of the RNeasy column after elution.

1: Dry down 5-10 µg of RNA in a speed-vac to 3 µl.

2: Add 1 µl of 0.4 M Na₂CO₃ pH 8.5. Vortex vigorously to resuspend any precipitated RNA.

3: Add 4 µl of dye solution, mix by vortexing and incubate for 1 h in the dark.

4: Add 92 µl of RNase-free water and purify RNA on an RNeasy column as described above. Use buffer RPE for the wash steps. Elute twice with 30 µl of RNase-free water. Check the membrane for colored speckles (see above).

5: Measure dye incorporation and RNA recovery by spectrophotometry: Dilute 4 µl of the eluate into 46 µl of water and analyze the sample in a spectrophotometer using a micro-cuvette. For Cy3-containing samples measure the absorbance at 260 and 550 nm and for Cy5 at 260 and 650 nm. Calculate the dye incorporation as follows:

$$\text{dye molecules per 1000 nt} = (A_{\text{dye}}/A_{260}) \cdot (9010 \text{ cm}^{-1}\text{M}^{-1}/\epsilon_{\text{dye}}) \cdot 1000$$

with $\epsilon_{\text{Cy3}} = 150,000 \text{ cm}^{-1}\text{M}^{-1}$ and $\epsilon_{\text{Cy5}} = 250,000 \text{ cm}^{-1}\text{M}^{-1}$.

The labeling reaction normally incorporates 25-50 dye molecules per 1000 nt.

V. Probe hydrolysis

1: Combine the labeled RNAs of a sample pair (~110 µl). Dry down RNA solution to 9 µl in a speed-vac. To avoid over-drying, take the tubes out of the speed-vac a few time during the drying procedure and vortex them thoroughly.
Add 1 µl of 10X fragmentation buffer (Ambion) and mix by vortexing. Incubate at 70°C for exactly 10 min. Vortex the tubes for a few seconds. Put tubes on ice and add 1 µl of stop buffer.

Note: This method generates RNA fragments <200 nt (peak at ~85 nt).

2: Add 20 µl of RNase-free water to the sample.

- Pre-spin a Spin-50 column in a microcentrifuge at 1000 g for 3 min. Empty collection tube.
- Add 500 µl of RNase-free water to the column and spin column in a microcentrifuge at 1000 g for 3 min.
- Discard collection tube and transfer column to an amber tube.
- Load RNA sample onto the center of the column. Spin column in a microcentrifuge at 1000 g for 3 min.

3: Continue with the hybridization protocol for RNA probes.

MATERIALS AND REAGENTS:

Invitrogen:

E.coli Ribonuclease H (Cat. No. 18021071); 120 units, enough for 60 rxns
E.coli DNA Polymerase I (Cat. No. 18010025); 1000 units, enough for 25 rxns
Superscript II (Cat. No. 18064071); 4x10,000 units, enough for 200 rxns
dNTP set (100 mM) (Cat. No. 10297018)

Ambion:

Megascript T7 kit (Cat. No. 1334), enough for 40 rxns
Fragmentation reagents (Cat. No. 8740), enough for 200 rxns
Aminoallyl-UTP (Cat. No. 8437), enough for 33 rxns

Amersham:

Cy3 Mono-Reactive Dye Pack (Cat. No. PA23001)
Cy5 Mono-Reactive Dye Pack (Cat. No. PA25001)

Eppendorf:

Phase Lock Tubes, Gel Light (200), 1.5 ml tubes (Cat. No. 0032 007.961)

Qiagen:

RNeasy Mini Kit (250) (Cat. No. 74106)

Sigma:

Linear Polyacrylamide (Cat. No. 5-6575)

USA Scientific:

Spin-50 Mini-Column (Cat. No. 1415-1602)

Various sources:

T7dT primer (PAGE purified):

5' – TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG TTT
TTT TTT TTT TTT TTT TTN N–3'

Phenol:chloroform:isoamyl alcohol (25:24:1).

SOLUTIONS:

5x Second Strand Buffer (store at -20°C):

100 mM Tris-HCl pH 6.9

450 mM KCl

23 mM MgCl₂

50 mM (NH₄)SO₄

0.75 mM β-NAD⁺

0.1 M Na₂CO₃ pH 8.5:

Prepare fresh buffer every 3-4 weeks. Make up with RNase-free water.

Phosphate Wash Buffer:

Prepare: 1 M K₂HPO₄ and 1 M KH₂PO₄ solutions using RNase-free water.

Mix 9.5 ml of 1 M K₂HPO₄ and 0.5 ml of 1 M KH₂PO₄ to generate 1 M KPO₄ pH 8.5 buffer.

For 100 ml of wash buffer mix:

0.5 ml 1 M KPO₄ pH 8.5

80 ml 100% ethanol

19.5 ml RNase-free water

Cy-dye solutions (store at -20°C protected from light):

Resuspend the dried dye of one vial in 73 µl of DMSO. Avoid repeated thawing of the dye solutions.

Microarray hybridization – RNA probes

The following is a protocol for the hybridization of dye-labeled RNA to microarrays. This protocol works well for 70mers (Operon) printed on poly-lysine or on Corning GAP slides. The amount of hybridization solution is sufficient for a 22 mm X 40 mm Lifterslip.

I: Slide Hybridization

1: Dry down purified probes in a speed-vac. Resuspend pellet in 5 µl 10 mM EDTA. Make sure that no precipitate is left in the sample.

2: Pre-heat the bottom part of a hybridization chamber to ~55°C. Clean a Lifterslip in 95% ethanol and dry it with a Kimwipe. Remove any dust from the Lifterslip as well as from the array with compressed air. Place the Lifterslip on the array.

3: Heat up sample at 80°C for 1 min.

- 4:** Add 50 µl of Ambion SlideHyb Buffer #1 (pre-warmed for 15-30 min at 68°C; before use make sure that all precipitate has dissolved) and mix sample thoroughly by pipetting. Incubate for 2 min at 80°C.
- 5:** Spin tube for 30 sec to collect the content at the bottom. Incubate at 80°C for a few sec. Place the hybridization chamber with the slide on top of a styrofoam block. Apply probe (55 µl) carefully without capturing air bubbles underneath the LifterSlip.
- 6:** Distribute a total of 60 µl of 3XSSC in small drops left and right from the LifterSlip.
- 7:** Close the hybridization chamber and incubate it in a water bath for 16-24 h at 48°C. Cover the water bath with a lid or with aluminum foil, to avoid any light exposure (high temperatures accelerate destruction of the fluorophores).
- 8:** Put a glass dish and 1 liter of water in a 60°C incubator.

II: Slide Washes

- 1:** Prepare 1 liter of the first wash buffer: 1XSSC, 0.2% SDS, 1 mM DTT (make up with pre-warmed water). Put a slide rack in the bottom of the pre-warmed glass dish and add the wash buffer. When the temperature of the wash buffer is ~48°C, dip a slide into the wash buffer, turn it up-side-down and shake it gently until the cover slip comes off. Insert slide into the slide tray without taking it out of the wash solution. Cover the dish with aluminum foil and gently shake it for 10 min.
- 2:** Transfer the slide rack to a small glass dish with 0.5 liter of 0.1XSSC, 0.2% SDS, 1 mM DTT (room temp). Cover the slide chamber with aluminum foil and gently shake it for 10 min.
- 3:** Transfer the slide rack to a small glass dish with 0.5 liter of 0.1XSSC, 1 mM DTT (room temp). Shake dish gently for 1 min.
- 4:** Take the slides out of the last wash buffer and centrifuge them for 1 min at ~150 g. Scan the slides right away since the fluorescent signals (Cy5, in particular) may start to fade rapidly.

Alternatively, to avoid dye-fading, coat the slides immediately with DyeSaver. Since this reagent can cause significant background fluorescence particularly in the green channel, the coating should be thin and uniform. For this, put a horizontal shaker in a fume hood (Caution: the reagent is very toxic and flammable – keep the vial with DyeSaver closed whenever possible). Cover the platform of the shaker with paper towels. Tape the towels to the platform if necessary (they should not move much when the shaker is spinning at high rpms). Position a slide in the middle of the platform, array side up. The actual coating has to be done very quickly since the reagent will dry up very rapidly. Distribute 350 µl of DyeSaver over the entire length of the slide. Turn on shaker to high rpms for a few seconds until the entire array is covered with DyeSaver. Quickly lift slide up to a vertical position and gently tap the end of the slide onto a paper towel to get rid of as much excess DyeSaver as possible. Let the slide dry for a few minutes in an upright position.

MATERIALS AND REAGENTS

Ambion: SlideHyb Buffer #1 (Cat. No. 8861)

Erie Scientific: LifterSlip (e.g. Cat. No. 22x40I-2-4710)

Genishere: DyeSaver (Cat. No. Q100300)

20xSSC: 3 M NaCl (175.3g)
0.3 M Na-citrate (88.2g)
add H₂O to 1 l and adjust pH to 7 with NaOH

10% (w/v) SDS